Published in Protist 161, issue 1, 116-121, 2010
which should be used for any reference to this work

The Environmental Clade LKM11 and *Rozella* Form the Deepest Branching Clade of Fungi

Enrique Lara[a,b,c,d,1], David Moreira[a], and Purificación López-García[a]

[a]Unité d'Ecologie, Systématique et Evolution, UMR CNRS 8079, Université Paris-Sud, bâtiment 360, 91405 Orsay Cedex, France
[b]WSL, Swiss Federal Research Institute, Ecosystem Boundaries Research Unit, Wetlands Research Group, Station 2, CH-1015 Lausanne, Switzerland
[c]école Polytechnique Fédérale de Lausanne (EPFL), Laboratoire des Systèmes écologiques, Station 2, CH-1015 Lausanne, Switzerland
[d]University of Neuchâtel, Institute of Biology, Soil & Vegetation Laboratory, CH-2009 Neuchâtel, Switzerland

Previous environmental surveys of eukaryotic diversity using the small subunit ribosomal RNA (SSU rRNA) gene have revealed many clone sequences that branch near the base of Fungi. In this work, we demonstrate that many of these sequences, including those of the environmental clade LKM11, form a monophyletic and strongly supported group that also includes two sequences derived from the parasitic genus *Rozella*. This novel clade, called here "Rozellida", is the deepest branch of true fungi so far identified, and appears to be extremely diverse in the environment.

Key words: environmental clades; fungi; LKM11 group; phylogeny; *Rozella*; SSU rRNA gene.

Introduction

During the last twenty years, environmental DNA surveys based on the analysis of small subunit ribosomal RNA genes (SSU rRNA) have revealed the existence of a wide microbial diversity, including many deep-branching prokaryotic clades lacking cultured members (Barns et al. 1996; Hugenholtz et al. 1998; López-García and Moreira 2008). More recently, similar studies on eukaryotic diversity have also shown that, although eukaryotes are far more diverse than previously thought, most novel sequences determined from environmental surveys actually cluster into major acknowledged eukaryotic "kingdoms" (Berney et al. 2004). To date, in contrast to the situation in prokaryotes, only very few environmental sequences could not be associated with any of the major eukaryotic kingdoms, for example the clade DH148-5-EKD18 (López-García et al. 2001; López-García and Moreira 2008). At the next lower taxonomic level, however, the situation is far from being resolved. Several groups of environmental sequences within alveolates, heterokonts, and a variety of other eukaryotic phyla cannot be linked to any described organism. For instance, within the heterokonts or stramenopiles, thirteen undescribed clades initially detected in marine surface waters have been reported, i.e. the so-called MAST groups (Massana et al. 2004; Massana and Pedrós-Alió 2008). Alveolate

[1]Corresponding author; fax +41 21 693 39 13
e-mail enrique.lara@epfl.ch (E. Lara).

sequences from environmental Groups I and II, which are highly abundant in marine clone libraries (Epstein and López-García 2008; Guillou et al. 2008), have only recently had their taxonomic identification recognised as Syndiniales. Group I includes the genera *Duboscquella* (Harada et al. 2007), *Ichtyodinium* (Mori et al. 2007) and a Red Plasmodial parasite (Skovgaard and Daugbjerg 2008), whereas Group II comprises sequences of the genera *Syndinium*, *Amoebophrya* and *Hematodinium* (Skovgaard et al. 2005). All of the known species of Group I and II alveolates are parasites/parasitoids of marine organisms (radiolaria, dinoflagellates but also copepods and teleost fishes) and this lifestyle is likely extensive to the two groups.

Opisthokonts also include a group of environmental sequences to which no described organism has yet been assigned, the LKM11 group. LKM11 was the first representative sequence, retrieved from a freshwater engineered system ten years ago (Van Hannen et al. 1999). This sequence has since been shown to represent a group of unidentified eukaryotes proposed on the basis of SSU rRNA environmental surveys. At the time when this pioneering study was first published, the small number of eukaryotic sequences present in the database did not allow the authors to place these sequences within the Opisthokonts or the Amoebozoa with significant phylogenetic support. Later, Berney et al. (2004) demonstrated that these sequences were probably related to Fungi. However, the exact phylogenetic position of the LKM11 group with respect to the Fungi remained unclear.

In this work, we analysed the phylogenetic position of the group LKM11 and relatives from the public database together with clones affiliated with this group retrieved from an environmental survey of eukaryotic SSU rRNA sequences from a peat bog. We show the existence, after the inclusion of representative sequences of major deep-branching fungal groups, of a large and diverse monophyletic clade of SSU rRNA sequences including LKM11 and *Rozella* as either the most basal branch of Fungi or their closest known sister group.

Results and Discussion

In the course of a general study of peat bog protist diversity, we identified a few sequences related to the environmental group LKM11; two of these were retrieved from peat samples (PRS2_4E_31 and PRS2_4E_06) and one from water samples (PR5_4E_71). We incorporated these sequences in an alignment of SSU rRNA gene sequences comprising both environmental and culture-derived sequences from several fungal clades, especially from the most basal-branching groups. Only nearly full-length SSU rRNA gene sequences were included in the alignment and long branching sequences were excluded based on initial phylogenetic analyses. We then constructed phylogenetic trees that were rooted using other opisthokont groups as outgroup sequences, including Nucleariida, Metazoa, Mesomycetozoa and Choanoflagellata. Bayesian and maximum likelihood phylogenetic analyses of these data revealed the existence of a robust clade composed mostly of environmental sequences and supported with posterior probability, PP, of 0.96 and bootstrap value, BV, of 100%. The only exceptions to this are sequences from two isolates belonging to the genus *Rozella*: *R. allomycis* and *Rozella* sp. ex. *Rhizoclosmatium* (see Fig. 1). The LKM11 (AJ130849) sequence branched within this group, as well as other environmental sequences classified in other studies as related to it (Lefranc et al. 2005; Luo et al. 2005; Šlapeta et al. 2005; Van Hannen et al. 1999). Given the high statistical support in our phylogenetic analyses for the *Rozella*/LKM11 clade, we propose here to name this group “Rozellida”, in reference to the genus *Rozella*. We suggest keeping this name between quotation marks until morphological and/or ultrastructural synapomorphies are defined to diagnose and validate this entire group. “Rozellida” appears to be the sister clade of all other true fungi (PP of 1, BV of 97%). The Nucleariida, the group of phagotrophic eukaryotes that has been found to be the closest relatives of Fungi (Steenkamp et al. 2005), appears as the only deeper branch in holofungi than “Rozellida”.

All 26 described species in the genus *Rozella* are parasites. The host range of *Rozella* spp. comprises primarily Chytridiomycetes, Blastocladiomycetes and Oomycetes (“zoosporic fungi”). However, the fact that *Rozella coleochaetis* has been found in the filamentous green alga *Coleochaete* (Sparrow et al. 1965) suggests additional potential hosts. Each *Rozella* morphospecies seems to be specific to a restricted number of hosts (Held 1981). Their life cycle comprises a uniflagellate motile stage that allows them to disperse in search of a new host, and a trophic wall-less intracellular stage, which develops inside a host cell (Held 1981). At this point, the parasite is amoeboid and phagocytoses the organelles of the

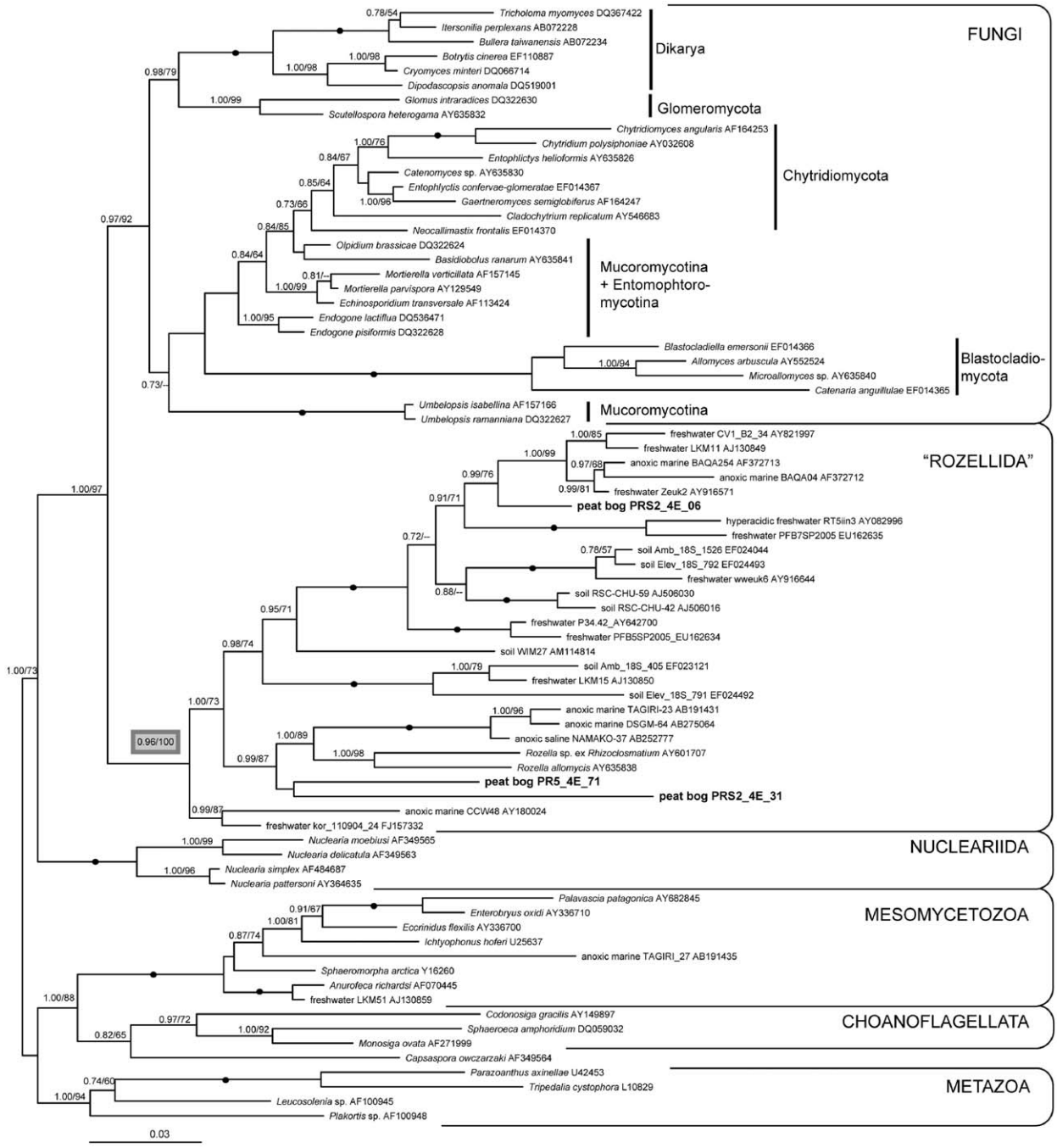

Figure 1. Phylogenetic tree illustrating the position of LKM11 within fungi, as obtained with a Bayesian approach, upon an alignment of 109 sequences and 1176 characters. Numbers at nodes represent, respectively, Bayesian posterior probabilities (PP) and maximum likelihood bootstrap values (BV). Black circles represent full support for the node with the two approaches (PP=1.00; BV=100%). Only support values above 0.70 PP and 50% BV are indicated. A – sign indicates that the node was not recovered with maximum likelihood. Clones obtained in this study are shown in bold.

host (Powell 1984); no filamentous growth (formation of hyphae/rhizoid) has been observed in this genus. Subsequently, the parasite eventually induces the host to create a cell wall that will surround the parasite's future sporangium; the parasite never builds its own cell wall. Flagellated cells are produced within these walled sporangia (Held 1980). Alternatively, *Rozella* spp. can produce resting forms, which are surrounded by a thick wall. Although *Rozella* species have been reported to be parasites of soil and freshwater hosts mainly, a marine species has been also described, *Rozella marina*, which is associated with the chytrid *Chytridium polysiphoniae* (Sparrow 1936).

Originally, the genus *Rozella* was placed into the Chytridiomycota, and specifically within the Spizellomycota, where it was classified inside the family Olpidiaceae (Adl et al. 2005). Its placement inside chytrids was partly justified on the basis of plesiomorphic characters, such as the existence of a flagellated stage (James et al. 2006a). The family Olpidiaceae originally comprised the genera *Olpidium*, *Entophlyctis*, *Rhizophlyctis* and *Rozella*. These genera were grouped together on the basis of the behaviour of the nucleus during development (Barr 1990). However, molecular phylogeny based on SSU rRNA and several other markers invalidated this taxon, and placed *Rozella* spp. at the base of the fungal tree with strong support (in agreement with our analysis), whereas *Olpidium* branched with zygomycetes and *Rhizophlyctis* occupied an unstable position within chytrids (Hibbett et al. 2007; James et al. 2006a). The phylogenetic position of "Rozellida" found in our analyses raises the question whether these organisms should be considered true Fungi or not. In our tree, the support for having "Rozellida" outside the remaining fungi is strong (PP=0.97%, BV=92%, Fig. 1). Following Adl et al.'s list of synapomorphies that define Fungi (Adl et al. 2005), *Rozella* spp. should logically not be included within this taxon since true Fungi are defined as organisms that have lost phagocytosis, which is retained in the trophic stage of *Rozella* spp. (Powell 1984). However, whether other uncultured members of "Rozellida" also have a phagotrophic stage in their life cycle remains to be determined. As the Nucleariida, the closest known sister group of Fungi are phagotrophic, it is probable that this feeding strategy was the ancestral state for the Fungi. Consequently, the secondary loss of phagocytosis might not be a good diagnostic character. Other features of *Rozella* spp. advocate for their placement into Fungi: flagellated zoospores with fungal-like ultrastructure of the flagellar apparatus (Held 1975), thick-walled resting spores and penetration of the host via a germ tube (Held 1973). Further prospecting on more representatives of the "Rozellida" will be crucial for circumscribing the true fungi.

The original environments from which the clones considered in this study derived were mainly freshwater (like LKM11; Van Hannen et al. 1999) but also soil (such as Elev_18S_791; Lesaulnier et al. 2008) and marine sediments (for instance TAGIRI 23; Takishita et al. 2005). Many were retrieved from anoxic or potentially anoxic environments (like, for instance, CCW48 and BAQA254; Dawson and Pace 2002; CV1_B2_34; Šlapeta et al. 2005). This corresponds to the environments where *Rozella* spp. have been reported, but also to those inhabited by some of their potential hosts. Chytrids, for instance, are typical inhabitants of freshwater and soil systems, although some species can be found in marine environments as well (Küpper and Müller 1999). Additionally, many chytrid species live in anoxic conditions, as some are facultative anaerobes with obligate fermentation (Emerson and Natvig 1981). Conversely, although the most extensively sampled environment in DNA surveys is clearly the oceanic euphotic zone (Epstein and López-García 2008), we did not find any sequence related to LKM11 in published molecular surveys from this environment, suggesting that their potential chytrid and oomycete hosts may be rare or even absent in surface marine waters. Furthermore, the frequent presence of long branches inside the "Rozellida" agrees with the hypothesis that this group might be composed to a large extent (if not entirely) of parasites, as parasites, in particular intracellular ones, very often have accelerated evolutionary rates (Itoh et al. 2002).

However, it would be premature to draw any conclusion on the lifestyle and ecology of these organisms based only on environmental sequences. Further work is required to identify and characterise additional members of this group. Their phylogenetic position as the most basal branch of fungi, or as their sister group, depending on how fungi are to be defined, gives them special importance. This is further reinforced by the suggestion, on the basis of protein-coding genes, that Microsporidia could be related to *Rozella allomycis* (James et al. 2006b). This hypothesis is particularly attractive given the likely parasitic nature of the two groups. Both groups would also share the absence of a cell wall when

invading host cells, so that the plasma membrane of the parasite makes direct contact with the cytoplasm of the host cell. However, no sequences from this group were included in our analyses, because microsporidian SSU rRNA sequences have extremely long branches being one of the most striking examples of a long branch attraction artefact in eukaryotic trees (Philippe and Adoutte 1998). An increase in the sampling of "Rozellida" taxa sampled and the sequencing of an adequate number of conserved genes for those species could help resolve this important and long-standing debate on the origins of Microsporidia, as well as resolving a number of open questions on the early evolution of fungi.

Methods

Sample collection, DNA extraction, PCR and cloning: Samples were taken at the Praz-Rodet peat bog, located in Switzerland (46°33′N; 06°10′E; altitude 1041 m). Water was sampled in a pool where vegetation was dominated by half submerged *Sphagnum cuspidatum*, with presence of *Drosera rotundifolia*. The water was prefiltered at 50 μm, and the biomass was gathered on a 0.2 μm nitrocellulose GTTP Millipore filter. DNA was extracted from the filters as described elsewhere (Lara et al. 2009). In addition, peat was also collected for DNA extraction, which was performed using a MoBio Power Soil™ DNA extraction kit (Carlsbad, CA USA) following the manufacturer's instructions. DNA was amplified using the eukaryotic-specific primers EK-82F (GAAACTGCGAATGGCTC) and EK-1492R (CACCTACGGAAACCTTGTTA). Subsequently, two clone libraries per sample were built. PCR reactions were carried out in 25 μl of reaction buffer containing 1 μl DNA template (~1-5 ng), 1.5 mM $MgCl_2$, dNTPs (10 nmol each), 20 pmol of each primer, and 1 U Taq DNA polymerase (Promega). PCR reactions were performed under the following conditions: 35 cycles (denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, extension at 72 °C for 2 min) preceded by 2 min denaturation at 94 °C, and followed by 8 min extension at 72 °C. Amplicons were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and transformed into *Escherichia coli* TOP10' One Shot cells (Invitrogen) according to the manufacturer's instructions. Clone inserts were amplified with vector T7 and M13R primers, and inserts of the expected size were sequenced directly using vector primers by Cogenics (Meylan, France). The obtained sequences can be retrieved from GenBank under accession codes FJ976648–-FJ976650.

Sequence and phylogenetic analyses: LKM11 clone sequences were aligned with a representation for fungal SSU rRNA sequences obtained from the GenBank data base using MUSCLE v. 3.6 (Edgar 2004). After excluding ambiguously aligned positions and gaps, the resulting alignment was subjected to Bayesian phylogenetic analysis with the software MrBayes v. 3.1.2 (Huelsenbeck and Ronquist 2001). The model chosen was GTR (Lanave et al. 1984; Rodríguez et al. 1990) with the number of invariable sites being estimated, and a gamma-shaped distribution of variable sites with four rate categories (GTR+Γ+I). Four chains were run up to 1,000,000 generations from a random starting tree well beyond convergence (the value of Ln L stabilised around -15000; PSRF parameter=1.006). Trees were sampled every 1000 generations. The first 5000 trees were discarded as the burn in. In addition, a maximum likelihood analysis was carried out using the program TREEFINDER (Jobb et al. 2004), applying a GTR+Γ+I model of nucleotide substitution (Rodríguez et al. 1990). All necessary parameters were estimated from the data sets. Bootstrap values were calculated from 1000 replicates.

Acknowledgements

This work was supported by an ATIP Plus grant of the French Centre National de la Recherche Scientifique (CNRS), section "Dynamique de la biodiversité" to P.L.G. E.L. benefited from an ATIP-associated CNRS research assistant contract.

References

Adl SM, Simpson AGB, Farmer MA, Andersen RA, et al. (27 authors) (2005) The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J Eukaryot Microbiol **52**: 399–451

Barns SM, Delwiche CF, Palmer JD, Pace NR (1996) Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. Proc Natl Acad Sci USA **93**:9188–9193

Barr DJS (1990) Phylum Chytridiomycota. In Margulis L, Corliss JO, Melkonian M, Chapman DJ (eds) Handbook of Protoctista. Jones & Bartlett, Boston, pp 454–466

Berney C, Fahrni J, Pawlowski J (2004) How many novel eukaryotic kingdoms? Pitfalls and limitations of environmental DNA surveys. BMC Biol **2**:1–13

Dawson SC, Pace NR (2002) Novel kingdom-level eukaryotic diversity in anoxic environments. Proc Natl Acad Sci USA **99**:8324–8329

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res **32**:1792–1797

Emerson R, Natvig DO (1981) Adaptation of Fungi to Stagnant Waters. In Wicklow DT, Carroll GC (eds) The Fungal Community, its Organization and Role in the Ecosystem. Marcel Dekker, New York, pp 109–128

Epstein SS, López-García P (2008) 'Missing' protists: a molecular prospective. Biodivers Conserv **17**:261–276

Guillou L, Viprey M, Chambouvet A, Welsh RM, Kirkham AR, Massana R, Scanlan DJ, Worden AZ (2008) Widespread occurrence and genetic diversity of marine parasitoids belonging to Syndiniales (Alveolata). Environ Microbiol **10**:3349–3365

Harada A, Ohtsuka S, Horiguchi T (2007) Species of the parasitic genus *Duboscquella* are members of the enigmatic Marine Alveolate Group I. Protist **158**:337–347

Held AA (1973) Encystment and germination of parasitic chytrid *Rozella allomycis* on host hyphae. Can J Bot **51**:1825–1835

Held AA (1975) Zoospore of *Rozella allomycis*-ultrastructure. Can J Bot **51**:1825–1836

Held AA (1980) Development of *Rozella* in *Allomyces*: A single zoospore produces numerous zoosporangia and resistant sporangia. Can J Bot **58**:959–979

Held AA (1981) *Rozella* and *Rozellopsis*: Naked endoparasitic fungi which dress up as their hosts. Bot Rev **47**:451–515

Hibbett DS, Binder M, Bischoff JF, et al. (66 authors) (2007) A higher-level phylogenetic classification of the Fungi. Mycol Res **111**: 509–547

Hugenholtz P, Goebel BM, Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol **180**:4765–4774

Huelsenbeck JP, Ronquist F (2001) MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics **17**:754–755

Itoh T, Martin W, Nei M (2002) Acceleration of genomic evolution caused by enhanced mutation rate in endocellular symbionts. Proc Natl Acad Sci USA **99**:12944–12948

James TY, Letcher PM, Longcore JE, Mozley-Standbridge SE, Porter D, Powell MJ, Griffith GW, Vilgalys R (2006) A molecular phylogeny of the flagellated fungi (Chytridiomycota) and description of a new phylum (Blastocladiomycota). Mycologia **98**:860–871

James TY, Kauff F, Schoch CL, et al. (70 authors) (2006b). Reconstructing the early evolution of fungi using a six-gene phylogeny. Nature **443**: 818–822

Jobb G, Von Haeseler A, Strimmer K (2004) TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol Biol **28**:4–18

Küpper FC, Müller DG (1999) Massive occurrence of the heterokont and fungal parasites *Anisolpidium*, *Eurychasma* and *Chytridium* in *Pylaiella littoralis* (Ectocarpales, Phaeophyceae). Nova Hedwigia **69**:381–389

Lanave C, Preparata G, Saccone C, Serio, G (1984) A new method for calculating evolutionary substitution rates. J Mol Evol **20**: 86–93

Lara E, Moreira D, Vereschchaka A, López-García P (2009) Pan-oceanic distribution of new highly diverse clades of deep-sea diplonemids. Environ Microbiol **11**:47–55

Lefranc M, Thénot A, Lepère C, Debroas D (2005) Genetic diversity of small eukaryotes in lakes differing by their trophic status. Appl Environ Microbiol **71**:5935–5942

Lesaulnier CC, Papamichail D, McCorkle SR, Ollivier B, Skiena S, Taghavi S, Zak DR, van der Lelie D (2008) Elevated atmospheric CO_2 affects soil microbial diversity associated with trembling aspen. Environ Microbiol **10**:926–941

López-García P, Rodríguez-Valera F, Pedrós-Alió C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature **409**:603–607

López-García P, Moreira D (2008) Tracking microbial biodiversity through molecular and genomic ecology. Res Microbiol **159**:67–73

Luo QW, Krumholz LR, Najar FZ, Peacock AD, Roe BA, White DC, Elshahed MS (2005) Diversity of the microeukaryotic community in sulfide-rich Zodletone Spring (Oklahoma). Appl Environ Microbiol **71**:6175–6184

Massana R, Balagué V, Guillou L, Pedrós-Alió C (2004) Picoeukaryotic diversity in an oligotrophic coastal site studied by molecular and culturing approaches. FEMS Microbiol Ecol **50**:231–243

Massana R, Pedrós-Alió C (2008) Unveiling new microbial eukaryotes in the surface ocean. Curr Opin Microbiol **11**:213–218

Mori K, Yamamoto K, Teruya K, Shiozawa S, Yoseda K, Sugaya T, Shirakashi S, Itoh N, Ogawa K (2007) Endoparasitic dinoflagellate of the genus *Ichthyodinium* infecting fertilized eggs and hatched larvae observed in the seed production of leopard coral grouper *Plectropomus leopardus*. Fish Pathol **42**:49–57

Philippe H, Adoutte A (1998) The Molecular Phylogeny of Eukaryota: Solid Facts and Uncertainties. In Coombs G, Vickerman K, Sleigh M, Warren A (eds) Evolutionary Relationships among Protozoa. Chapman & Hall, London, pp 25–56

Powell MJ (1984) Fine structure of the unwalled thallus of *Rozella polyphagi* in its host *Polyphagus euglenae*. Mycologia **76**:1039–1048

Rodríguez F, Oliver JL, Marin A, Medina JR (1990) The general stochastic model of nucleotide substitution. J Theor Biol **142**:485–501

Skovgaard A, Massana R, Balagué V, Saiz E (2005) Phylogenetic position of the copepod-infesting parasite *Syndinium turbo* (Dinoflagellata, Syndinea). Protist **156**:413–423

Skovgaard A, Daugbjerg N (2008) Identity and systematic position of *Paradinium poucheti* and other *Paradinium*-like parasites of marine copepods based on morphology and nuclear-encoded SSU rDNA. Protist **159**:401–413

Šlapeta J, Moreira D, López-García P (2005) The extent of protist diversity: insights from molecular ecology of freshwater eukaryotes. Proc R Soc Lond B Biol Sci **272**:2073–2081

Steenkamp ET, Wright J, Baldauf SL (2005) The protistan origins of animals and fungi. Mol Biol Evol **23**:93–106

Sparrow FK (1936) Biological observations on the marine fungi of Woods Hole waters. Biol Bull **70**:236–263

Sparrow FK, Paterson RA, Johns RM (1965) Additions to the phycomycete flora of the Douglas Lake region. V. New or interesting fungi. Pap Michigan Acad Sci **50**:115–123

Takishita K, Miyake H, Kawato M, Maruyama T (2005) Genetic diversity of microbial eukaryotes in anoxic sediment around fumaroles on a submarine caldera floor based on the small-subunit rDNA phylogeny. Extremophiles **9**:185–196

Van Hannen EJ, Mooij WM, van Agterveld MP, Gons HJ, Laanbroek HJ (1999) Detritus-dependent development of the microbial community in an experimental system: qualitative analysis by denaturing gradient gel electrophoresis. Appl Environ Microbiol **65**:2478–2484